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13. ABSTRACT (Maximum 200 Words)
Fibroblast growth factor binding protein (FGF-BP) releases immobilized FGFs from the extracellular matrix and can function as an angiogenic switch molecule in cancer. We have determined that FGF-BP is upregulated in a portion of breast cancers and this upregulation is correlated with increased expression of β-catenin. In this grant we hypothesized that β-catenin can initiate angiogenesis in mammary carcinoma through FGF-BP. The aims were 1) to study the expression of FGF-BP in mammary tumorigenesis progression of the APC/+ mouse and 2) to determine the mechanism of regulation of FGF-BP b the APC/β-catenin signaling pathway in breast cancer. To date, we have shown a positive correlation of upregulation of β-catenin expression and FGF-BP in breast and other tumors in the APC/+ mice. We have also shown that β-catenin can directly induce FGF-BP gene expression through a transcriptional mechanism and that a TCF site in the FGF-BP promoter is responsible for a major portion of this effect. To further study the nature and specificity of this interaction, we performed gel shift assays and determined that the TCF site at -1030 in the FGF-BP promoter is necessary for this interaction.

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INTRODUCTION

Breast cancer is dependent on angiogenesis for growth and malignant progression. Without angiogenesis, the tumors would not have the proper nutrients for growth and would be limited in their ability to enter the circulatory system and metastasize [1]. The process of angiogenesis is controlled and regulated by a number of different protein factors. FGF-2 is one of the most potent of the pro-angiogenic factors [2]. FGF-2 is normally sequestered in the extracellular matrix (ECM) and can be released by enzymatic cleavage of heparin sulfate proteoglycans or by the fibroblast growth factor binding protein (FGF-BP) [3]. FGF-BP can reversibly bind to FGF-2 and release it from the ECM [3]. Previous work from our laboratory indicate that FGF-BP acts as an angiogenic factor and is expressed in a subset of breast cancers as well as in squamous cell carcinoma and colon carcinoma as indicated by histological analysis of human tumor samples (Ray et al appendix manuscript). We examined the expression of FGF-BP in the mammary tumors of the Min/+ mouse. This mouse model has a germline mutation in the adenomatous polyposis coli (APC) gene. A second somatic hit in the second allele of this gene produces a non-functional APC protein [4]. This mutation results in overexpression of the oncogene beta-catenin. In this study we examine the regulation of FGF-BP1 by beta-catenin in breast cancer.

BODY

We have previously shown that FGF-BP is expressed in the mammary tumors of the Min/+ mouse. In this report and the previous report work was accomplished toward the goals stated in Aim 2 (To study the mechanism of regulation of FGF-BP by the APC/Beta-catenin signaling pathway in breast cancer) are discussed. First, it was necessary to confirm that FGF-

BP was indeed regulated by beta-catenin. Although FGF-BP was coexpressed with beta-catenin in mammary tumors of the Min/+ mouse, this only confirms spatial correlation of the two proteins. In order to establish a direct relationship between beta-catenin and FGF-BP, a series of *in vitro* assays were undertaken using two different breast cancer cell lines. The MDA-MB-468 breast cancer cell line expresses endogenous FGF-BP and low levels of beta-catenin. This cell line was used to test whether increases in beta-catenin levels would effect the endogenous expression of FGF-BP. To induce higher levels of beta-catenin, the MDA-MB-468 cells were treated with lithium chloride. Lithium inhibits glycogen synthase kinase-3beta (GSK-3beta) a negative regulator of beta-catenin. Upon treatment of MDA-MB-468 with Lithium chloride, which resulted in a subsequent increase of cytoplasmic and nuclear beta-catenin, there was a 3-fold induction of FGF-BP1 mRNA levels (appendix manuscript). **Therefore, beta-catenin can regulate the endogenous FGF-BP1 gene product in breast cancer cell lines.**

As stated in Aim 2- experimental series #2, the possibility that beta-catenin regulates FGF-BP at the transcriptional level has been examined. To determine whether FGF-BP regulation by beta-catenin occurs at the promoter, we transiently co-transfected the SK-BR-3 breast cancer cell line with a 1Kb portion of the FGF-BP promoter (-1060/+62 FGF-BP-luciferase) and a wild-type beta-catenin expression vector (appendix manuscript). We found that beta-catenin is able to activate the promoter up to 3.5 fold in breast cancer cells (appendix manuscript). Furthermore, E-cadherin, which sequesters beta-catenin, was co-transfected with FGF-BP and beta-catenin into breast cancer cells and was able to reverse the beta-catenin-mediated induction of FGF-BP (appendix manuscript). These results verify that FGF-BP is a direct target of beta-catenin and that it occurs at a transcriptional level. Many beta-catenin target genes are transcriptionally regulated via a TCF-site (5'-A/T A/T CAAAG-3') [5]. In order to

determine the relevant promoter regions of FGF-BP for beta-catenin regulation, a series of FGF-BP promoter fragments were cloned into the PGL3 empty vector. These fragments were sequential 5'-deletions of the promoter beginning from a 1 Kb piece. Some of these constructs were created using restriction enzyme digestions while the others were made with the QuikChange Site-Directed Mutagenesis Kit (Stratagene). These progressive 5' deletion constructs of FGF-BP +/- beta-catenin were transiently transfected into the SK-BR-3 breast cancer cell line (Fig 4 in appendix manuscript). Deletion of the region –1060 to –118 caused a loss of about 60% of the inductive effect and further deletion of –93 to –77 caused complete loss of the remaining induction (Fig 4 in appendix manuscript) In the upstream region a large portion of the β-catenin inductive effect is mediated by a TCF site at –1030 (Fig 4 in appendix manuscript). Through these luciferase reporter assays we showed that the FGF-BP promoter contains a regulatory region that is necessary for promoter activation by β-catenin.

To further analyze this DNA-protein interaction we performed electrophoretic mobility shift (gel shift) assays in the breast caner cell line MDA468. Different regions of the FGF-BP promoter region were tested for interaction with b-catenin obtained from MDA468 cell lysates. To compete the interaction we used both specific and non-specific ^{32}P-labelled probes. As shown in Figure 1A (appendix), the radiolabelled probes taken from the wildtype promoter contained either the TCF site at –1030 (1030wt) or at –545 (545wt). To compete interaction of these probes with β-catenin, we used unlabelled 1030wt and 545wt probes as well a nonspecific competitor. Furthermore, in order to verify that specificity of the interaction occurs through the TCF site, additional probes were used from which the -1030 and -545 TCF sites were deleted (1030Δ and 545Δ).

As shown in Figure 1B (appendix), the interaction between 1030wt and β-catenin could be competed by even small amounts of 1030wt competitor whereas the 1030D competitor was not as effective. In fact the 1030D probe could only completely inhibit the interaction of 1030wt and β-catenin at high concentrations. These results indicate that the TCF site at –1030 of FGF-BP is necessary for specific interaction with β-catenin. The 545wt probe, which contained the other TCF site in the FGF-BP promoter, could also interact with β-catenin. In this case, however the 545wt and 545Δ probes could inhibit the interaction between the -545 TCF site and β-catenin to the same extent suggesting that the TCF site at –545 on the FGF-BP promoter is not necessary for β-catenin binding. The nonspecific competitor could not inhibit the DNA-protein interaction in either case indicating that the interaction between FGF-BP promoter and β-catenin is specific.

As indicated in our last report, deletion experiments of the non-TCF site located between –152 and –139 on the FGF-BP gene promoter did not give a consistent abrogation of the β-catenin inductive effect. However, we shoed that in cells where the β-catenin allele is knocked out, there is substantially less promoter activity than in the wild type cells (Fig 4 in Appendix manuscript).

Research Accomplishments

- Identification of a relevant genetically defined mouse model of breast cancer to study the expression pattern and function of the angiogenic factor FGF-BP.
    - We have identified FGF-BP as a novel target gene of the WNT/beta-catenin signaling pathway.

- We have identified a TCF site in the FGF-BP gene promoter that explains a significant portion of the β-catenin inductive effect. In addition more proximal regions of the gene promoter are also involved in regulation and are currently being studies for their role in the β-catenin induction of FGF-BP in breast cancer.
- We have shown that loss of a β-catenin allele causes substantial reduction in FGF-BP gene promoter activity.
- We have shown that the TCF site at –1030 on the FGF-BP promoter is necessary for the specific interaction with β-catenin.

Abstracts

Ray, R., Cabal-Manzano, R., Riegel, AT., Wellstein, A. The Angiogenic Factor Fibroblast Growth Factor Binding Protein (FGF-BP), a novel beta-catenin target gene. American Association of Cancer Research, New Orleans, LA (2001).

Papers: **Ray, R,** Cabal-Manzano-R, Moser A.R., Waldmann,T., Zipper,LM, Aigner,A, Byers,S.W. Riegel,A.T. and Wellstein A. Up-regulation of FGF-BP by β-catenin during colon carcinogenesis. Cancer Research 63:8085-8089, 2003

Conclusions

The goal of **Aim 2, experimental series #2** was to determine if FGF-BP1 is a target gene of the WNT/beta-catenin signaling pathway in breast cancer. Using a variety of *in vitro* techniques (transient transfections, northern blot analysis, western blot analysis, site-directed mutagenesis, etc.) we have confirmed that beta-catenin does regulate FGF-BP in breast cancer

cell lines as well as other tumor types. Because beta-catenin is overexpressed in human breast cancer [6] it may possibly act as an oncogene in this disease as it does in colon cancer. Our work presents the possibility that beta-catenin may influence tumor angiogenesis in breast cancer through FGF-BP. In the past year we have refined our analysis of the FGF-BP gene promoter and determined that a TCF site at –1030 contributes a major portion of the β-catenin effect. In addition more proximal regions of the promoter are highly involved in β-catenin regulation and these sites as well as the regulation of the TCF sites are currently under investigation in the laboratory.

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Appendix

Fig 1A. Competitor Probes used in gel shift assays.

1030wt: probe containing the TCF site at –1030 and flanking sequences on the FGF-BP gene promoter

1030Δ: probe containing only the flanking sequences but not the TCF site at –1030 on the FGF-BP gene promoter.

545wt: probe containing the TCF site at –1030 and flanking sequences on the FGF-BP gene promoter

545Δ: probe containing only the flanking sequences but not the TCF site at –1030 on the FGF-BP gene promoter.

NS: non-specific probe

Fig 1B. Gel shift assay using β-catenin and different regions of the FGF-BP gene promoter. Triangles indicate increasing concentrations of competitor probe.

Figure 1

A

1030wt: CAAATGTGTGTTTTCAAAGTATACAACTTAAG

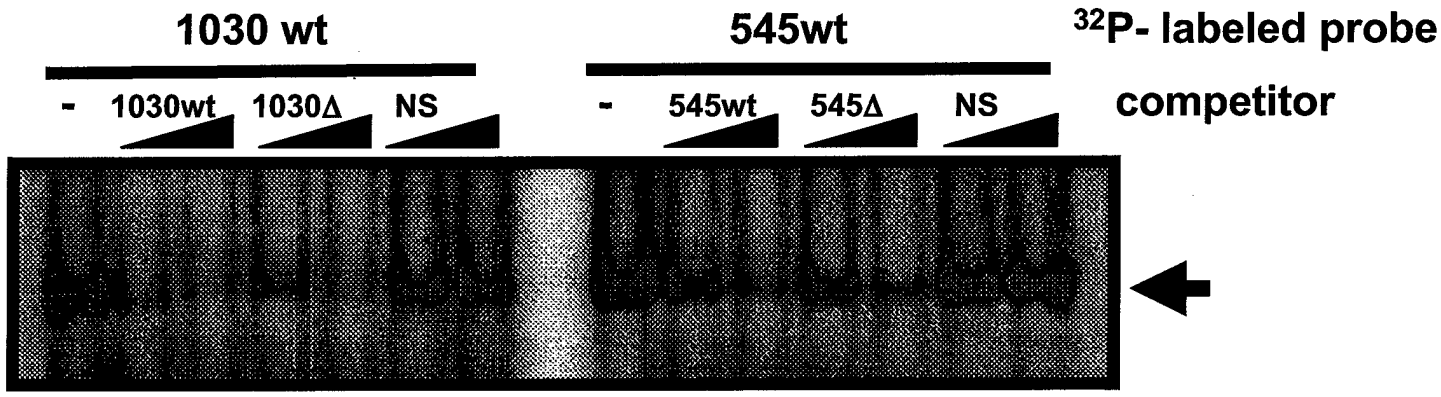
1030Δ: CAAATGTGTGTT**TATACAACTTAAG

545wt: CAGTCACCCATTCAACAAAGATTTATTAGAAGTGG

545wt: CAGTCACCCATTC**ATTTATTAGAAGTGG

NS: Non-specific probe

B



[CANCER RESEARCH 63, 8085–8089, December 1, 2003]

Advances in Brief

Up-Regulation of Fibroblast Growth Factor-Binding Protein, by β-Catenin during Colon Carcinogenesis

Ranjan Ray,[1] Rafael Cabal-Manzano,[1] Amy R. Moser,[2] Todd Waldman,[1] Laurie M. Zipper,[1] Achim Aigner,[1] Stephen W. Byers,[1] Anna T. Riegel,[1] and Anton Wellstein[1]

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Abstract

Fibroblast growth factor-binding protein (FGF-BP) releases immobilized FGFs from the extracellular matrix and can function as an angiogenic switch molecule in cancer. Here we show that FGF-BP is up-regulated in early dysplastic lesions of the human colon that are typically associated with a loss of adenomatous polyposis coli and up-regulation of β-catenin. In addition, FGF-BP expression is induced in dysplastic lesions in ApcMin/+ mice in parallel with the up-regulation of β-catenin. Also, in cell culture studies FGF-BP is induced by β-catenin through direct activation of the FGF-BP gene promoter. We conclude that FGF-BP is a target gene of β-catenin.

Introduction

We have shown previously that the secreted binding protein FGF-BP[3] can act as a chaperone for locally stored FGFs and enhance their angiogenic activity, thus allowing FGF-BP to serve as an angiogenic switch molecule in cancer (1). Consistent with a role for FGF-BP in cancer, ribozyme-targeted depletion of FGF-BP from human colon cancer or squamous cell carcinoma cells showed a rate-limiting role for FGF-BP in tumor growth and angiogenesis (1). Hence, we proposed that this molecule is one of the "angiogenic switch" mechanisms required for malignant progression (1–3).

We found that FGF-BP is expressed at high levels in the murine gut during embryonic development, down-regulated in the adult (4, 5), but expressed at high levels in some colon cancer tissues and cell lines (1). To evaluate regulation of FGF-BP during colon carcinogenesis we initiated a series of studies with normal and pathological colon biopsies to determine at what stage of transformation the gene is up-regulated. Here we report that FGF-BP expression is highly up-regulated in dysplastic lesions, *i.e.*, early on during colon carcinogenesis. These early lesions are associated with mutations in β-catenin, and/or a loss of function of the *APC* tumor suppressor gene has been identified in >80% of sporadic colon carcinomas (6). To assess the possible contribution of the loss of APC to FGF-BP up-regulation, we used a well-defined murine model, the B6 ApcMin/+ mouse, which carries one mutant APC allele and develops polyps on loss of the residual wild-type APC allele (7, 8). In this model we found that FGF-BP and β-catenin expression was induced in polyps, as well as in a rare ACF, the earliest discernible stages of transformation (9). Furthermore, cell culture studies show that increases in endogenous β-catenin by treatment with LiCl result in a significant increase in FGF-BP mRNA levels, and cotransfection assays demonstrate transcriptional activation of the FGF-BP gene promoter by β-catenin through T-cell factor (TCF) sites. We conclude that FGF-BP is a novel target gene of the Wnt/β-catenin pathway.

Materials and Methods

Tissue Samples, Immunohistochemistry, and *in Situ* Hybridization. Paraffin-embedded archival tissues were provided by the tumor tissue core facility of the Lombardi Cancer Center with patient identifiers removed. Dr. Moser (University of Wisconsin) and Drs. Herfarth and Schoelmerich (University of Regensburg) provided samples from mouse models. H&E stains of serial sections were reviewed by a pathologist to verify the diagnosis. The categorization followed Dukes' classification (10). Serial sections of 4 μm were used for FGF-BP protein staining or FGF-BP mRNA detection by *in situ* hybridization. The *in situ* hybridization protocol was described earlier using human and mouse FGF-BP riboprobes (4, 11) that were digoxigenin-labeled using the DIG RNA labeling mix (Roche). For the immunohistochemistry a rabbit polyclonal anti-FGF-BP antibody (diluted 1:150 in 2% BSA/PBS) was used. As described earlier, this antibody recognizes murine, rat, and human FGF-BP in archival tissue sections (5, 11). A β-catenin monoclonal antibody was purchased from Transduction Laboratories (Lexington, KY). Stained cells were divided into three grade levels: grade 0, negative (absence of color); grade 1, moderately stained with an obvious brown color; and grade 2, vividly stained dark brown. A tissue section was considered as negative when <30% of the same morphological structure (normal mucosa, dysplasia, or cancer) showed any color (grade 0; Ref. 12). All of the others were scored as positive in the analysis. In a subset of samples angiogenesis was assessed after CD31 staining using light microscopy at ×200 in areas containing the highest number of capillaries or hot spots as described earlier (13).

Cell Culture, Transfections, and Reporter Assays. The cell lines CaCo-2 (colon cancer), SKBR3, and MDA-MB468 (breast cancer) were from the American Type Culture Collection (Springfield, VA). The HCT-116 cell line with somatic cell knockout of the activated β-catenin allele was provided by Dr. Todd Waldman (Georgetown University; Ref. 14). The cells were maintained in DMEM with 10% FBS at 37°C and 5% CO_2. Twenty-four h before transfection, cells were seeded in 12-well plates at a density of 1×10^5 cells/well in DMEM +10% FBS. With CaCo-2 cells, for each well 0.75 μg of DNA constructs and 10 μl of LipofectAMINE reagent were combined with 200 μl of OPTI-MEM1 (Life Technologies, Inc.) and incubated for 30 min at room temperature. Appropriate amounts of OPTI-MEM1 were added to the solution to bring the volume to 1 ml, and the mixture was placed on cells for 3 h at 37°C. The cells were then washed twice with Iscove's Modified Medium (IMEM, Life Technologies, Inc.) and incubated in DMEM +10% FBS for 18 h. For SKBR3 cells, DNA constructs were mixed in a 1:2 ratio with FuGENE (Roche) reagent in serum-free IMEM and incubated at room temperature for 30 min. The FuGENE/DNA solution was then added to the cells with DMEM +10% FBS medium and incubated at 37°C for 18 h. Transfection efficiency was determined by cotransfection with 4.0 ng of the *Renilla* luciferase reporter vector pRL-CMV (Promega). After the 18-h incubation, cells were lysed in 100 μl of Passive Lysis buffer (Promega). Ten μl of the cell extract was assayed for firefly and *Renilla* luciferase activity with the Dual-Luciferase Reporter assay system (Promega). FGF-BP promoter constructs (−1060/+62, −118/+62, −93/+62, and −77/+62) were cloned into the pGL3 vector as described previously (15). Two consensus TCF sites (5′-A/T

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Requests for reprints: Anton Wellstein, Lombardi Cancer Center, Georgetown University, Washington, DC 20057. Phone: (202) 687-3672; Fax: (202) 687-4821; E-mail: wellstea@georgetown.edu.

[3] The abbreviations used are: FGF-BP, fibroblast growth factor binding protein; APC, adenomatous polyposis coli; FBS, fetal bovine serum; ACF, aberrant crypt focus; VEGF, vascular endothelial growth factor; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

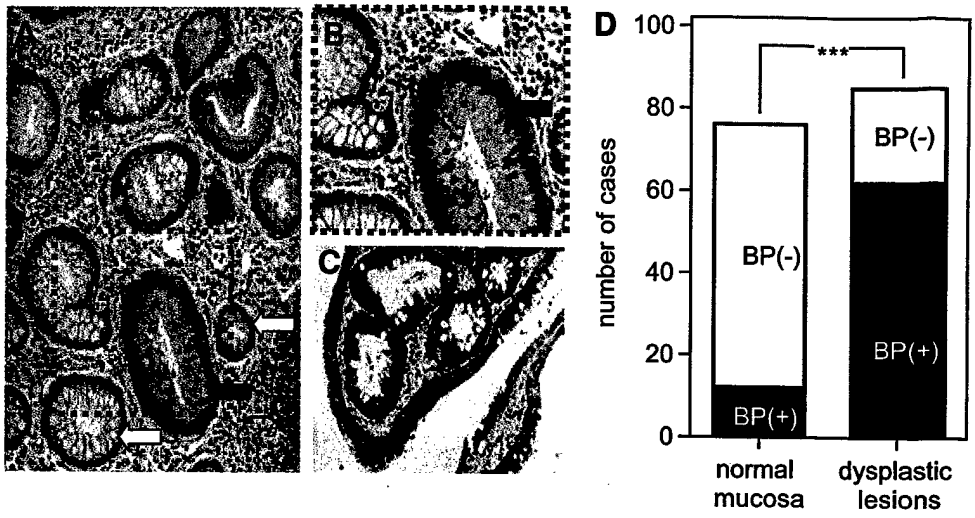

Fig. 1. FGF-BP1 expression in human colorectal samples. *A*, immunohistochemistry using a polyclonal antibody against FGF-BP1 shows FGF-BP1 protein expression in colonic crypts. Normal colon crypts (*open arrows*) and a dysplastic crypt (*closed arrow*) are indicated. *B*, magnification of the region indicated in *A* by the dotted frame. *C*, *in situ* hybridization for FGF-BP1 mRNA of a colon polyp with dysplastic crypts. *D*: frequency of FGF-BP1 protein expression using 161 different biopsies from patients with normal epithelium and dysplastic lesions. ***, $P < 0.0001$, χ^2.

A/T CAAAG-3′) located at −1030 and at −545 were deleted by PCR-based mutagenesis using the following primers: forward primers 1030-del-F: 5′-CAA ATG TCT GTT TAT ACA ACT TAA GAC CC-3′ and 545-del-F: 5′-CAG TCACCC ATT CAT TTA TTG AGA GTG G-3′; reverse primers 1030-del-R: 5′-GGG TCT TAA GTT GTA TAA ACA GAC ATT TG-3′ and 545-del-R 5-CCA CTC TCA ATA AAT GAA TGG GTG ACT G-3′. All of the constructs were sequenced to confirm mutations. SKBR3 cells were transfected with 100 ng of luciferase cDNA, 0.1 ng Renilla, and 300 ng of pCDNA3 or 300 ng pCDNA3-β-catenin using Fugene 6 transfection reagent (Roche). Experiments were typically performed in duplicate and repeated as indicated in the legends to the figures. β-Catenin, E-cadherin, and Topflash expression constructs were described previously (16). The pcDNA3 cloning vector was purchased from Invitrogen.

Northern Analysis. MDA-MB468 cells were plated in 10-cm dishes and grown to 70% confluence in IMEM +10% FBS 24 h before treatment. The cells were treated with LiCl (30 mM) + inositol or with NaCl (30 mM) + inositol dissolved in 10 ml of IMEM +10% FBS. Sixteen h after initiation of treatment total RNA was isolated using the RNA STAT-60 protocol (RNA STAT-60; Tel-Test, Friendswood, TX). Thirty μg of total RNA were run on a 1.2% formaldehyde-agarose gel. Blotting and hybridization with the human FGF-BP probe were performed as described previously (15).

Data Analysis. The Prism/GraphPad program was used for data analysis. *Ps* < 0.05 were considered significant.

Results

FGF-BP Is Up-Regulated during the Initiation of Human Colon Carcinogenesis. We had observed previously that FGF-BP is highly expressed during murine gut development and is down-regulated in the adult mouse (4). FGF-BP is also highly expressed in some human colon cancer cell lines and tissues (1). The stages of malignant progression toward colon cancer have been well delineated, and we hypothesized that analysis of colon biopsies of different pathological stages could indicate possible genetic alterations associated with the up-regulation of FGF-BP. A survey of human colon biopsy material (Fig. 1) showed that FGF-BP expression was detectable in 12 of 76 histologically normal appearing samples. In contrast with this, a high portion of the samples with moderate to severe dysplasia expressed FGF-BP (62 of 85; $P < 0.0001$ normal *versus* dysplastic mucosa; χ^2 test). Interestingly, even individual dysplastic crypts (closed arrow in Fig. 1*A*) located within otherwise normal mucosa (open arrow in Fig. 1) show expression of FGF-BP. It is of note that the increased expression of FGF-BP in dysplastic lesions coincides with a significant increase in blood vessel density from 80 ± 7 to 154 ± 9 vessels/field as measured by CD31 staining in the lamina propria (normal mucosa *versus* severe dysplasia; $P < 0.001$, *t* test).

To assess whether FGF-BP would be up-regulated by all of the pathological alterations in the gut, we studied a series of biopsy samples from different stages of inflammatory bowel disease. Only 3 of 26 samples from patients with ulcerative colitis ($n = 18$) or Crohn's disease without apparent dysplasia ($n = 8$) showed detectable FGF-BP expression. This frequency of expression was not different from the normal mucosa controls ($P > 0.05$, χ^2). Because inflammatory bowel disease does not induce FGF-BP expression, we hypothesized that the up-regulation of FGF-BP at the onset of colon epithelial malignant transformation may be due to an early genetic event, such as loss of the APC tumor suppressor function associated with the initiation of dysplasia. To address this question, we used the APC heterozygous B6 ApcMin/+ mouse (17) as an animal model system.

FGF-BP Expression in B6 ApcMin/+ Mouse Adenoma Coincides with Cellular Relocation and Increase in β-Catenin Protein. As a first step, we compared FGF-BP expression in the intestines of wild-type C57BL/6J mice relative to that in B6 ApcMin/+ mice. We found no differences in baseline expression of FGF-BP (data not shown). This suggests that the loss of function of one allele in the B6 ApcMin/+ mice is not sufficient to alter the signal toward FGF-BP expression. In the normal epithelium β-catenin is sequestered at the membrane and is rarely found in the cytoplasm or nucleus (Ref. 18; see Fig. 2*J*). When APC becomes defective in intestinal crypt cells of B6 ApcMin/+ mice, regulation of β-catenin is lost (17), and the epithelium progresses to early stages of malignancy. We used the accumulation of cytoplasmic and nuclear β-catenin in microscopic sections as a read-out for the loss of APC function, and probed serial sections of normal and adenoma tissues for both FGF-BP and β-catenin expression.

Tissues were harvested from animals between 91 and 132 days of age, and analyzed for β-catenin protein by immunohistochemistry and for FGF-BP mRNA by *in situ* hybridization. Dysplastic lesions within normal mucosal tissue (Fig. 2, *A* and *E*, darker H&E staining) showed an elevation of the β-catenin protein (Fig. 2, *B* and *F*, brown stain) and of FGF-BP mRNA (Fig. 2, *C* and *G*, dark blue stain). Fig. 2, *D* and H, show negative controls for FGF-BP detection. In a survey of tissues we found that 21 of 27 adenomas were strongly positive for FGF-BP (>30% of the adenoma surface area; Fig. 2, *C* and *G*). Adjacent normal intestine was also examined for FGF-BP expression, and only 5 of 19 samples showed any expression of FGF-BP ($P < 0.001$, normal *versus* adenomas, χ^2; Fig. 2*K*). On careful inspection of serial sections, we found a striking coincidence of expression of FGF-BP mRNA (Fig. 2, *C* and *G*) and of β-catenin protein (Fig. 2, *B* and *F*) in

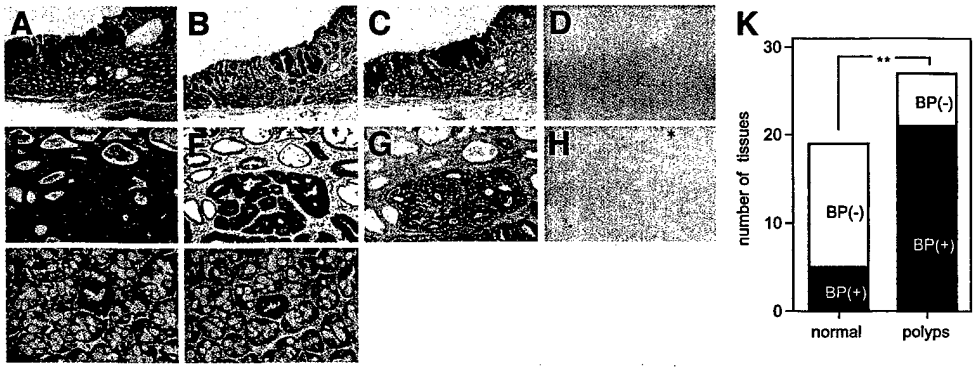

Fig. 2. FGF-BP and β-catenin expression in B6 ApcMin/+ mouse samples. Dysplastic lesions and adjacent normal mucosa is shown. In *E–H* (×6 magnification) * indicates the identical area in each slide for orientation purposes. *A* and *E*, H&E-stained section. Dysplastic lesions appear in *dark blue*. *B* and *F*, staining for β-catenin protein. *C* and *G*, *in situ* hybridization for FGF-BP mRNA. Antisense probe. Expression of FGF-BP is confined to the dysplastic region. *D* and *H*, FGF-BP sense probe as a negative control. *I* and *J*, staining for FGF-BP protein (*I*) and β-catenin protein (*J*) in a colon ACF that is surrounded by normal mucosa. *K*, frequency of FGF-BP expression in normal mucosa and polyps. **, $P < 0.001$, χ^2.

the same tissue areas. We also searched tissues for the earliest microscopically discernible stage of dysplasia, ACF, that is observed at a rate of 0.14 per mouse (19). Although we observed only a single ACF, there was coincidence of expression of the two genes in cells that formed this ACF in the midst of normal mucosal areas (Fig. 2, *I* and *J*).

In addition to the tissues derived from the ApcMin/+ model, we also examined sections from intestinal polyps in a dextran sulfate-induced model of inflammatory colon disease (20). No increase in FGF-BP expression was observed in this model (data not shown). This finding corroborates the lack of expression of FGF-BP in human clinical inflammatory bowel disease of different stages (see above). We concluded from these studies that FGF-BP expression is induced during the initiation of malignancy, and we hypothesized that this could occur is a result of the activation of the Wnt/β-catenin pathway.

Lithium Induces Endogenous FGF-BP mRNA Expression. To determine whether β-catenin is directly involved in the regulation of the FGF-BP gene, we examined whether lithium-induced β-catenin stabilization affects the levels of endogenous FGF-BP mRNA. Lithium inhibits glycogen synthase kinase-3β, a negative regulator of β-catenin (21). For the experiments we used MDA-MB468 breast cancer cells because they express detectable FGF-BP and show intact β-catenin regulation. The MDA-MB468 cells were treated for 16 h with LiCl and inositol, which prevents inositol 1,4,5-triphosphate depletion by LiCl (21). Cells treated with LiCl and inositol increased β-catenin protein levels and showed a 3-fold induction of FGF-BP mRNA as compared with control treatment (NaCl+inositol; Fig. 3). The NaCl+inositol control showed no significant effect on basal FGF-BP mRNA expression (data not shown). Thus, increasing the level of free β-catenin coincides with induction of endogenous FGF-BP mRNA, lending additional support to the notion of β-catenin as a regulator of FGF-BP expression.

β-Catenin Regulates FGF-BP Promoter Activity. To investigate whether the FGF-BP gene is a transcriptionally regulated target of β-catenin, we cotransfected a wild-type β-catenin expression vector with an FGF-BP expression vector containing the −1060/+62 fragment of the FGF-BP promoter upstream of a luciferase reporter. This 1060-bp FGF-BP promoter fragment contains numerous consensus transcription factor-binding sites that are necessary for the transcriptional activity of the promoter (15). In the CaCo-2 colon cancer cell line, which harbors an APC mutation and expresses endogenous FGF-BP, we found that β-catenin expression induces FGF-BP promoter activity up to 3-fold over basal levels (Fig. 4*A*). This increase in promoter activity in CaCo-2 cells is comparable with β-catenin induction of the TopFlash promoter, a known β-catenin sensitive promoter containing multimerized TCF sites (data not shown).

Because CaCo-2 cells harbor an APC mutation that results in high levels of endogenous β-catenin, we chose the SKBR3 breast cancer cell line as a model for a next series of studies, because these cells have no known mutations in the APC/β-catenin signaling pathway and express low basal levels of β-catenin. Thus, these cells provide a more sensitive system to observe the effects of β-catenin overexpression and dissect the pathway of induction. In these cells, the FGF-BP promoter activity is induced by expression of exogenous β-catenin (Fig. 4*B*). To determine whether this induction is a β-catenin-specific effect, we cotransfected E-cadherin, an adhesion molecule that binds to the internal armadillo repeats of β-catenin and functions as a dominant-negative regulator of β-catenin by preventing its translocation to the nucleus. Cotransfection with E-cadherin reversed β-catenin induction of the FGF-BP promoter (Fig. 4*B*). We also found that FGF-BP promoter activity of the full-length promoter −1060/+62 was reduced in isogenic HCT-116 colon cancer cells with somatic cell

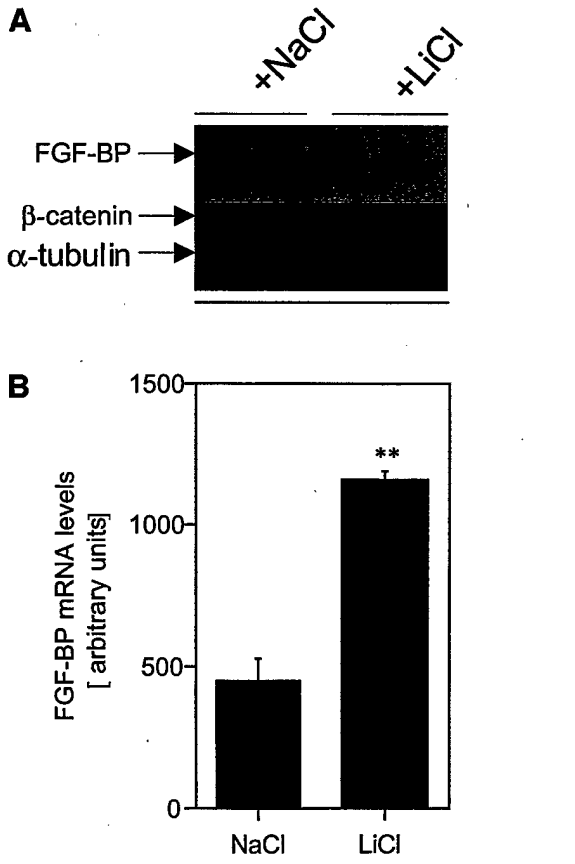

Fig. 3. Induction of FGF-BP mRNA by lithium chloride in MDA-MB468 cells. *A*, Northern blot for FGF-BP mRNA and Western blot for β-catenin. α-Tubulin is a loading control. Treatment was for 16 h. Duplicate samples were loaded. The experiment was repeated three times. *B*, levels of FGF-BP mRNA were quantified by phosphoimaging and corrected for loading by glyceraldehyde-3-phosphate dehydrogenase mRNA levels. **, $P < 0.01$ relative to NaCl treatment.

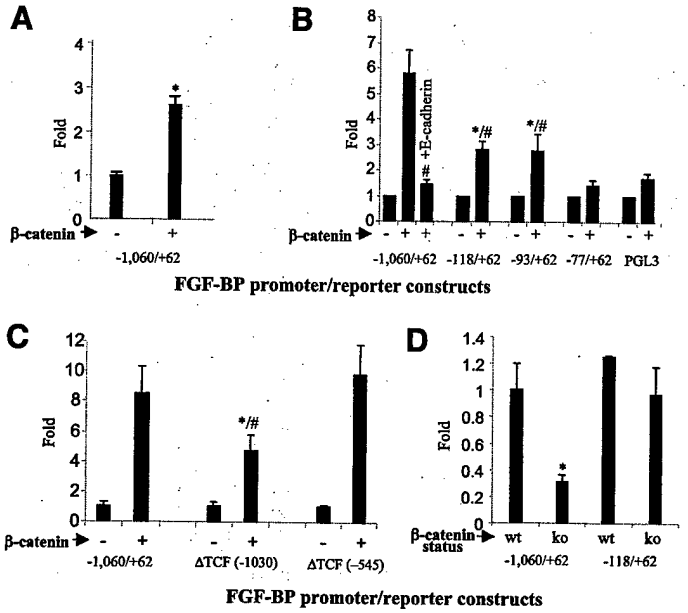

Fig. 4. Effect of β-catenin on FGF-BP promoter activity. CaCo-2 (*A*), SKBR3 (*B* and *C*), and HCT116 (*D*) cells were used. Different promoter/reporter constructs were transiently transfected as indicated plus either pcDNA or β-catenin ± E-cadherin and luciferase activity was measured after 18 h. E-cadherin was included with the −1060/+62 construct. Effect of the deletion of the consensus TCF site (5′-A/T A/T CAAAG-3′) on β-catenin induction of FGF-BP promoter activity. Means from three different experiments (*A* and *B*) and a representative experiment from two independent repeats (*C*) are shown; *bars*, ±SE. *, $P < 0.05$ relative to empty vector; #, $P < 0.05$ relative to −1060/+62. *D*, effect of somatic cell knockout of the mutated and activated β-catenin allele in HCT116 cells. The FGF-BP promoter/reporter constructs were transiently transfected (Fugene, Roche; 48 h) into the wild-type (*wt*) HCT116 colon cancer cell line that contains an activated β-catenin allele or into an isogenic knockout cell line (*ko*) in which the activated β-catenin allele was deleted (14). A representative experiment from two independent repeats with triplicate measurements each is shown. *, $P < 0.05$ relative to wild-type.

knockout of the activated β-catenin allele (14) as compared with promoter activity in wild-type HCT-116 cells (Fig. 4*D*). Taken together with the results of the β-catenin effects on endogenous levels of FGF-BP mRNA, these data indicate that β-catenin is a transcriptional regulator of the FGF-BP gene.

β-Catenin Regulatory Region in the FGF-BP Promoter. To identify the regions necessary for regulation of the FGF-BP promoter by β-catenin, we transfected SKBR3 cells with 5′ deletion constructs of the FGF-BP promoter/reporter constructs (15). β-Catenin had a minimal background effect (<2-fold) on luciferase activity of the pGL3-basic empty vector (Fig. 4*B*), similar to nonspecific background effects that we observed previously with this vector (15). Deletion from −1060 to −118 reduced the β-catenin induced promoter activity by >70%. An additional deletion to −93 had no effect on the induction of the promoter by β-catenin, but deletion to −77 negated all of the β-catenin induction of the promoter to background levels of the pGL3 vector (Fig. 4*B*). The experiments with the FGF-BP promoter/reporter constructs in the HCT-116 knockout cells, which have their activated β-catenin allele deleted (14), showed a significant reduction of constitutive promoter activity of the full-length construct. Constitutive activity of the −118/+62 construct, however, was not altered by the deletion of the activated β-catenin allele (Fig. 4*D*). This finding complements the different inducibility of the activity of these constructs by transfection of exogenous β-catenin in the SKBR3 cells (see Fig. 4*B*).

We found that the FGF-BP promoter contains two TCF consensus binding sites at −545 and −1030, and deleted these sites by PCR. Interestingly, only deletion of the distal TCF site at −1030 reduced β-catenin induction of promoter activity, whereas deletion of the proximal site at −545 had no significant effect (Fig. 4*C*). We conclude from these results that β-catenin induction of FGF-BP promoter activity involves regulatory regions in the distal promoter.

Discussion

In this study, we demonstrate that FGF-BP expression is up-regulated during early stages of human colon epithelial malignant progression, *i.e.*, during the earliest dysplastic stages associated with a loss of the APC tumor suppressor gene, and is induced in the intestinal adenomas of the B6 ApcMin/+ mouse. We show that FGF-BP expression coincides with the expression of β-catenin in early lesions, *i.e.*, adenomas and already an ACF in the B6 ApcMin/+ mouse, suggesting that FGF-BP lies downstream of the β-catenin signaling cascade. Finally, the studies in cultured cells show that β-catenin activates transcription from the FGF-BP promoter, thus providing evidence that this gene is a target of β-catenin.

Our previous analysis of FGF-BP expression in the developing mouse gut had shown that epithelial cells positioned at the bottom of the crypts express FGF-BP and that this expression is lost in cells maturing along the crypt/villus axis (4). More recently, positioning of epithelial cells along the crypt/villous axis and imposition of a crypt precursor phenotype was found associated with a gradient of β-catenin/TCF activity that shows its maximum at the bottom of the crypts and is reduced as cells differentiate during their migration up the crypt (22, 23). It is likely that the FGF-BP expression that we observed in histologically normal tissues represents staining of sections of the lower third of crypts and, hence, the region with high β-catenin activity. Also, FGF-BP expression may indicate a very early stage in the transition to dysplasia that is not yet manifest from the H&E staining. Fig. 1, *A* and *B*, shows such an example of dysplastic lesions with surrounding normal mucosa. Interestingly, the histologically normal crypts that do not express FGF-BP show some staining for the protein in sections that transverse the bottom of the crypt as indicated by the narrow opening of the crypt (compare the two crypts indicated by open arrows in Fig. 1*A*). With respect to distinct pathological alterations, the lack of FGF-BP expression in inflammatory human bowel disease and in the rodent animal model equivalent (20) suggest that inflammatory pathways in the colon do not lead to an up-regulation of FGF-BP either on their own or through cross-talk with the β-catenin signaling.

FGF-BP is an activator of growth factors in the FGF-family, and our studies lend support to the idea that the FGF family plays a role in the development of the early angiogenic phenotype in colon cancer. The induction of the angiogenic phenotype in colon cancer is a multifaceted process requiring the cooperation of numerous factors during the different steps of malignant progression. In a study of levels of FGF-2 and VEGF in serum samples from colon cancer patients, it was suggested that FGF-2 may act as an early inducer of the angiogenic phenotype (24). FGF-BP up-regulation in intestinal adenomas may indeed trigger this by providing the chaperone that can release the immobilized FGF-2. In support of this notion, we found increased angiogenesis coincident with FGF-BP expression in human colon dysplastic lesions. Other factors, such as VEGF, probably cooperate with FGF-2 to maintain the process of angiogenesis throughout the stages of tumor formation. VEGF expression is found in adenomas of the colon; however, unlike our findings with FGF-BP, increased VEGF expression levels are correlated with later stages of the disease, and VEGF expression is increased in carcinomas as compared with adenomas as well as in metastatic *versus* nonmetastatic colon cancer (25).

Not only does FGF-BP appear to be a novel proangiogenic target of β-catenin that is up-regulated at an early stage of premalignant lesions, it seems that its regulation is through areas of the FGF-BP

promoter that are not required for basal growth factor or TPA regulation of this gene in either squamous cell carcinoma or breast cancer cells (*e.g.,* see Refs. 15, 26). In fact, the regulation by EGF and TPA in these cell types involves an activator protein, a CAAT/enhancer binding protein β site, and an E-box repressor that are all situated downstream of −118 and that are activated predominantly through the p38/mitogen-activated protein kinase pathway. Therefore, an unexpected result was the involvement of the region between −1060 and −118 in the β-catenin regulation of FGF-BP. This also indicates that β-catenin is not activating the promoter via indirect activation of the mitogen-activated protein kinase signaling pathways. Examination of the 1-kb promoter region between −1060 and −118 for possible transcription factor recognition sites using Transfac analysis revealed two potential TCF/lymphoid enhancer factor (LEF) sites, which are known to be involved in β-catenin regulated gene transcription. Deletion of these sites showed that only the −1030 site contributes to β-catenin-induced promoter activation, whereas the site at −545 does not. Interestingly, there is also a perfect consensus site for REL/nuclear factor κB present in this region, which is of interest because β-catenin can interact directly with nuclear factor κB and might, thus, contribute in addition to regulation of the gene promoter (27). The second surprise of the FGF-BP gene promoter analysis was that the region between −93 and −77 was also required for full β-catenin induction of the promoter. We have demonstrated previously that this region harbors an SP1 binding site and can bind SP1 specifically. However, this site is not required for growth factor or TPA-regulated gene transcription (15). It remains to be determined whether this site acts cooperatively with upstream regulatory factors in the β-catenin induction of FGF-BP gene transcription. The Sp1 and Krueppel-like factors that bind to GC boxes are known to play a role in cell growth and tumor progression. However, their role in early events in colon carcinogenesis has not been defined.

In conclusion, β-catenin, one the most significant oncogenic proteins in colon cancer, has been implicated in several key steps of the path to malignancy. The cell cycle regulatory genes, c-Myc and cyclin-D1, have both been identified as targets of β-catenin. These two proteins were also found overexpressed in intestinal adenomas of the B6 ApcMin/+ mouse. Furthermore, β-catenin activates matrix metalloproteinase 7, an enzyme that plays a role in invasion and metastasis. Additionally, APC and E-cadherin, two proteins that are closely tied to β-catenin function, are important for induction of apoptosis and cell-cell adhesion, respectively. Our identification of FGF-BP as a direct target of β-catenin transcriptional activation suggests that β-catenin can also play a role in promoting the switch to the angiogenic phenotype observed early in the malignant progression of colon cancer.

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